Type 7 Adenylyl Cyclase-Mediated Hypothalamic-Pituitary-Adrenal Axis Responsiveness: Influence of Ethanol and Sex

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ABSTRACT

Although ethanol has been considered to be an anxiolytic agent, consumption of ethanol has also been shown to increase plasma adrenocorticotropin and glucocorticoids. The corticotrophin-releasing factor (CRF) receptor 1 α (CRF-R1) is a G protein-coupled receptor that activates adenylyl cyclase (AC), leading to adrenocorticotropin (and subsequently glucocorticoid) release into the circulation. There are nine members of the membrane-bound AC family, and the type 7 AC (AC7) is most sensitive to ethanol, which enhances the responsiveness of AC7 to G protein-coupled receptor activation. We determined the time course of ethanol's effect on plasma adrenocorticotropin and corticosterone levels in male and female AC7 transgenic (Adcy7^{huTG}) mice (in which AC7 is overexpressed in neural tissue) and AC7 heterozygous knockdown [Adcy7(+/-)] mice (in which AC7 is underexpressed in neural

tissue), and their respective littermate controls [wild type (WT)]. CRF-R1 mRNA and mRNA and protein for different forms of ACs were measured by using gene expression arrays, quantitative reverse transcription-polymerase chain reaction, and immunoblotting in pituitaries of all animals. Our results demonstrated increased levels of AC7 in pituitary of Adcy7^{huTG} mice and decreased levels in pituitary of Adcy7(+/-) mice compared with WT animals. Male and female Adcy7^{huTG} mice displayed higher plasma adrenocorticotropin and corticosterone levels than WT and/or Adcy7(+/-) mice after ethanol injection. Female mice displayed higher adrenocorticotropin and corticosterone levels after ethanol injection than males, regardless of genotype. The data provide evidence for an integral role of AC7 in the increase of plasma adrenocorticotropin and corticosterone levels during alcohol intoxication.

A well established, but sometimes overlooked, fact about the physiologic actions of ethanol is that even modest doses of ethanol in humans or other animals can increase circulating levels of adrenocorticotropin and cortisol or corticosterone (Tabakoff et al., 1978; Rivier et al., 1984; Thiagarajan et al., 1988; Ogilvie et al., 1997).

The mechanism by which ethanol increases circulating glucocorticoid levels has been traced to increases in adrenocorticotropin release (Rivier et al., 1984, 1996; Lee et al., 2004), and corticotrophin-releasing factor (CRF) has also been shown to be important for ethanol's actions. Thus, it was found that administration of anti-CRF serum totally abolished ethanol-induced release of adrenocorticotropin (Rivier et al., 1984). Whether ethanol was injected intraperitoneally or intracerebroventricularly, there was no increase

in circulating adrenocorticotropin if hypothalamic CRF was absorbed by preinjection of CRF antibodies (Lee et al., 2004).

A significant increase in CRF mRNA and CRF peptide release in primary hypothalamic cell cultures did occur from 30 min to 4 h after application of alcohol (25 mM) (Li et al., 2005). However, significant increases in adrenocorticotropin release in vivo have been reported within minutes and reached a peak as early as 15 min after systemic ethanol injection (Thiagarajan et al., 1988; Ogilvie et al., 1997; Lee et al., 2004). These data suggest that the early in vivo effect of ethanol on adrenocorticotropin release may not depend on an increase in CRF release. Taking these findings into account, one can hypothesize that ethanol can enhance CRF-stimulated adrenocorticotropin release by acting at the level of the CRF receptor, i.e., ethanol can increase signal transduction occurring in the presence of the activated CRF receptor.

It is well accepted that CRF receptor 1 α (CRF-R1) is a G_s-coupled receptor, and CRF binding to this receptor initiates signaling by activating adenylyl cyclase (AC) through G_s α (Wynn et al., 1985; Aguilera et al., 1986; Grigoriadis et al., 1993; De Souza, 1995). What was not investigated, until

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ABBREVIATIONS: AC, adenylyl cyclase; AC7, type 7 AC; AC9, type 9 AC; AUC, area under the curve; AVP, arginine vasopressin; CRF, corticotrophin-releasing factor; CRF-R1, CRF receptor 1 α ; PKC, protein kinase C; WT, wild type; ANOVA, analysis of variance; qRT-PCR, quantitative real-time polymerase chain reaction; FDR, false discovery rate.

the recent work of Antoni et al. (2003), was the identity of the isoforms of AC to which CRF-R1 couples. Using pituitary corticotrophs, Antoni et al. (2003) demonstrated coupling of CRF-R1 to AC9, but they also hypothesized that, during stress, CRF-R1 coupling to AC9 in the corticotroph was switched to coupling to AC7. This switch was proposed to be a result of the actions of arginine vasopressin (AVP), acting through the AVP1b receptor (V1b receptor) to produce changes in receptor/effecter (CRF-R1/AC) coupling through a PKC-mediated phosphorylation. We have previously shown that PKC δ -mediated phosphorylation of AC7 increases AC7 responsiveness to $G_{\text{S}\alpha}$ (Nelson et al., 2003), whereas $G_{\text{S}\alpha}$ -stimulated AC9 activity is significantly attenuated by PKC-induced phosphorylation (Cumbay and Watts, 2004). Further attenuation of AC9 activity can be produced by the AVP/ G_i /phospholipase C/inositol trisphosphate-mediated release of intracellular Ca^{2+} (Antoni et al., 1998), whereas AC7 is Ca^{2+} -insensitive (Antoni et al., 1998; Mons et al., 1998). Because ethanol exerts its effects on $G_{\text{S}\alpha}$ -AC7 coupling via ethanol-induced phosphorylation of AC7 by PKC δ (Nelson et al., 2003), one can propose that ethanol would be able to increase CRF-R1 and $G_{\text{S}\alpha}$ -mediated cAMP signaling by promoting the phosphorylation of AC7, in a manner similar to what was suggested for vasopressin.

One way in which the role of AC7 can be better defined with regard to ethanol's actions on adrenocorticotropin and corticosterone release in animals is to genetically manipulate the expression of AC7 in pituitary of mice by creating transgenic (Adcy7^{huTG}) and knockdown [Adcy7(+/-)] mice. We have created and used such mice for these studies of ethanol's actions.

Materials and Methods

Animals. Experiments were performed on 70- to 100-day-old mice. AC7 transgenic (Adcy7^{huTG}) mice overexpress human AC7 in the brain (Yoshimura et al., 2000) and had been backcrossed to C57BL/6 mice for 13 generations at the time of these experiments. AC7 heterozygous "knockdown" mice, in which one copy of the AC7 gene was disrupted [Adcy7(+/-)] (Hines et al., 2006), were backcrossed to C57BL/6 mice for 12 generations. (Disruption of both copies of the AC7 gene produced death of the fetus.) The respective littermates were used as controls (WT). The animals were housed in a colony room with a 12-h light/12-h dark cycle with controlled temperature (23°C) and humidity (40%). Access to food and water was ad libitum. All efforts were made to minimize any potential suffering, and all experimental procedures were approved by the University of Colorado Denver Institutional Animal Care and Use Committee. To desensitize mice to handling, animals were handled once a day for a week before experiments. Animals were singly housed 1 day before the experiment. Ethanol was administered, and blood was collected starting at approximately 8 AM.

Microarray Analysis. Naive male and female Adcy7(+/-), Adcy7^{huTG}, and WT animals were used for microarray analysis. Animals were rapidly anesthetized with CO_2 and decapitated, and whole pituitary glands were quickly removed and placed in RNAlater RNA Stabilization Reagent (QIAGEN, Valencia, CA). Total RNA from two pooled whole pituitaries (Adcy7^{huTG} and WT animals), or single pituitaries [Adcy7(+/-) and WT animals], was extracted by using the RNeasy Micro Kit (QIAGEN) following the manufacturer's instructions. Gene expression data were obtained by using the Affymetrix (Santa Clara, CA) GeneChip Mouse Genome 430 version 2.0 arrays as described previously (Tabakoff et al., 2008) and stored on the PhenoGen Informatics web site (<http://phenogen.ucdenver.edu>). Data were subjected to quality control (Saba et al., 2006) and nor-

malization by using the Robust Multichip Average/Robust Multichip Analysis method (Irizarry et al., 2003) after probes of poor sequence integrity were removed. The expression levels (mRNA) of corticotrophin-releasing hormone receptor 1 (*Crhr1*), $G_{\text{S}\alpha}$ protein (*Gnas*), proopiomelanocortin (*Pomc*), and AC1-AC9 (*Adcy1-Adcy9*) genes in the pituitary glands of male and female Adcy7(+/-), Adcy7^{huTG}, and corresponding WT animals were analyzed by one-way ANOVA with pairwise contrasts for the four comparisons of interest: female Adcy7(+/-) versus WT, female Adcy7^{huTG} versus WT, male Adcy7(+/-) versus WT, and male Adcy7^{huTG} versus WT. A false discovery rate (FDR) (Benjamini and Hochberg, 1995) was used to adjust for multiple comparisons within each pairwise contrast.

Quantitative Reverse Transcription Real-Time Polymerase Chain Reaction. Quantitative real-time polymerase chain reaction (qRT-PCR) (Prism 7900 Sequence Detection System; Applied Biosystems, Foster City, CA) was used, in addition to microarray analysis, to quantitate the expression of the transgene (human *ADCY7*) mRNA and endogenous (mouse *Adcy7*) mRNA in anterior pituitary from male and female Adcy7^{huTG} and Adcy7(+/-) mice and their wild-type littermates.

Sequence-specific TaqMan probes and primer sets for human *ADCY7* were designed by using PrimerExpress software (Applied Biosystems); probes and primers for mouse *Adcy7* were designed by Applied Biosystems. The probes and primers are described elsewhere (Hines et al., 2006).

Anterior pituitaries from three to four animals were pooled, and total RNA was extracted by using an RNeasy Plus Mini-Kit (QIAGEN). Double-stranded cDNA was synthesized from total RNA and used to obtain biotin-labeled cRNA by an in vitro transcription reaction. All samples were assayed in triplicate on a single plate for human *ADCY7* and mouse *Adcy7*. After correction for the endogenous control, mouse glyceraldehyde-3-phosphate dehydrogenase (*Gadph*) mRNA, the relative quantities of each transcript were calculated as described by Livak and Schmittgen (2001). Data were analyzed by two-way ANOVA (sex and genotype) and post-hoc contrasts when appropriate.

Immunoblotting for AC7 Protein. AC7 protein was measured as described elsewhere (Hines et al., 2006) with modifications. In brief, antiserum was generated in rabbits against a peptide sequence within the C1b region of AC7 (ETHVPNGRRPKSVQRHRRTC), and the antibodies recognized both human and mouse AC7 protein. Mice were killed with CO_2 , and the whole pituitary gland was dissected. Glands were homogenized by using a Polytron (KINEMATICA AG, Lucerne, Switzerland) in 10 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA, 0.25 M sucrose, and protease and phosphatase inhibitor cocktails (Sigma-Aldrich, St. Louis, MO) and centrifuged at 1600g (10 min, 4°C). The protein concentration of the particulate-containing supernatant was determined by the BCA method (Pierce Chemical, Rockford, IL). Protein (45 μg per lane) was mixed with NuPAGE LDS sample buffer and NuPAGE reducing agent (Invitrogen, Carlsbad, CA), separated on 4 to 12% Bis-Tris polyacrylamide gels (NuPAGE gels; Invitrogen) and transferred to nitrocellulose membranes. The blots were blocked with StartingBlock (phosphate-buffered saline) blocking buffer (Thermo Fisher Scientific, Waltham, MA) and incubated with primary antibody to AC7 (1:3000) and monoclonal antibody to β -tubulin (1:10,000; BD Biosciences PharMingen, San Diego, CA), followed by goat anti-rabbit IgG (1:10,000) and goat anti-mouse IgG (1:30,000) coupled to horseradish peroxidase. Protein bands were visualized with enhanced chemiluminescence reagent (PerkinElmer Life and Analytical Sciences, Waltham, MA). After exposure to X-ray film, protein bands were quantitated by image analysis using Quantity One software (version 4.5.1; Bio-Rad Laboratories, Hercules, CA). In many instances (but not all), AC7 appears as a doublet (molecular mass 100 and ~80 kDa; see Fig. 2). We had previously noted a less prominent doublet in hippocampal tissue (Hines et al., 2006). Where a doublet was noted, both bands were included in the

densitometric analysis. Results were calculated as densitometric volume of the appropriate bands, and levels of AC7 protein were normalized based on β -tubulin levels. Every gel contained protein from WT, *Adcy7^{huTG}*, and *Adcy7(+/-)* mice. On each blot, the densitometric ratio values (AC7/ β -tubulin) for the *Adcy7^{huTG}* and *Adcy7(+/-)* mice were converted to percentage of WT ratios on that blot (data from male and female animals were analyzed separately). These percentage values were averaged across blots for male and female mice. Data were analyzed by two-way ANOVA (sex and genotype) with a random effect of subject, followed by post-hoc contrasts when appropriate.

Effect of Ethanol on Plasma Adrenocorticotropin and Corticosterone Levels. All experiments were performed between 8:00 and 9:30 AM. Ethanol was injected intraperitoneally at a dose of 2.25 g/kg as a 15% solution (diluted in saline). At 0, 15, 30, 45, and 75 min after ethanol injection or 15 min after saline injection (as a control for the effect of injection per se) mice were anesthetized by exposure to CO₂ for 10 s and decapitated, and trunk blood was collected in ice-chilled Eppendorf tubes containing 20 μ l of 5% EDTA. Blood was centrifuged to separate plasma. The separated plasma was stored at -80°C until analysis. Corticosterone concentrations were determined in 25 μ l of plasma by using a commercially available enzyme immunoassay kit (Diagnostic Systems Laboratories, Inc., Webster, TX) following the manufacturer's directions. Adrenocorticotropin was measured in 100 μ l of plasma by using an adrenocorticotropin enzyme-linked immunosorbent assay kit (ALPCO Diagnostics, Windham, NH). Blood ethanol levels were determined (in 20- μ l samples from the same mice used for the endocrine assays) by head-space gas chromatography, using a Varian, Inc. (Palo Alto, CA) 3800 gas chromatograph as described previously (Tabakoff et al., 1978).

Statistical Analysis of Injection Stress on Adrenocorticotropin Levels. A three-way ANOVA was used to analyze the effect of injection stress on adrenocorticotropin levels across sexes and genotypes (sex $\times$ genotype $\times$ time). Data were included from all three genotypes and both sexes at baseline, i.e., immediately after injection with ethanol (0 time) and 15 min after injection with saline. Sex effects and sex-specific genotype effects were examined at baseline and after saline injection. In addition, the injection effect (adrenocorticotropin levels 15 min after saline injection minus adrenocorticotropin levels at baseline) was examined separately by sex and genotype. Effect *P* values were adjusted for multiple comparisons by using the Bonferroni multiple comparison procedure.

Statistical Analyses of Ethanol Effects on Adrenocorticotropin and Corticosterone Levels. Before analysis, the data were tested for equality of variances across groups created by the combination of sex, genotype, and time, using Levene's test for homogeneity

(Levene, 1960). This test indicated a significant difference among group variances ($F = 3.25$, $P < 0.0001$ for adrenocorticotropin; $F = 3.92$, $P < 0.0001$ for corticosterone) that was not resolved by commonly used transformations such as log or square root. Therefore, data were analyzed by using a three-way ANOVA model that allows heterogeneous variances, as executed by using the MIXED procedure in SAS version 9.2 (SAS Institute, Cary, NC). General significance (differences associated with genotype and/or sex) within each time point was then tested for each outcome (adrenocorticotropin or corticosterone levels). For time points that indicated a significant effect, genotype, sex, and other specific effects were tested by using appropriate linear contrasts, and specific group differences of interest were estimated. *P* values for the general significance at each time point were adjusted for multiple comparisons by using the Bonferroni multiple comparison procedure.

Areas under the curve (AUC) were calculated from 0 to 75 min by using the mean values for each genotype and sex combination. The variance of the AUC was calculated by using the serial sacrifice method described by Nedelman et al. (1995). To determine the effect of ethanol on AUC, each AUC was calculated assuming no change in baseline levels over time. For this calculation, the mean variance and sample size from time 0 were used as baseline data to correct values at 15, 30, 45, and 75 min after injection. AUC values were compared pairwise among genotypes within sexes and between sexes within genotypes, using *t* tests with the degrees of freedom estimated by using the Satterthwaite approximation assuming unequal variances outlined in Nedelman et al. (1995).

Data are presented as mean and standard errors calculated by using the least-squares estimation procedure within the ANOVA. *P* values ≤ 0.05 were considered statistically significant throughout.

Results

Microarray Analysis of Whole Pituitary Gland. Analysis of microarray data (Table 1) revealed no significant differences in pituitary expression (mRNA levels) of corticotrophin-releasing hormone receptor 1 (*Crhr1*), $G_s\alpha$ protein (*Gnas*), or proopiomelanocortin (*Pomc*) between *Adcy7^{huTG}* and WT, or *Adcy7(+/-)* and WT, animals of either sex, after correction for multiple comparisons. Although probe sets for mouse *Adcy1-Adcy9* were present on arrays, only mRNAs for *Adcy2*, *Adcy3*, *Adcy6*, and *Adcy7* were found to be present in pituitary glands, based on the criterion that at least one probe set for the transcript was called "present" on all arrays by the Affymetrix MAS 5.0 algorithm (Affymetrix, 2001).

TABLE 1

Measures of pituitary levels of mRNA for adenylyl cyclase isoforms, CRF-1, $G_s\alpha$ protein, and proopiomelanocortin by microarray analysis

The percentage change in all cases represents the amount of difference in the quantity of mRNA for a particular transcript found in whole pituitary of the genetically modified strains versus the respective WT mice. All measurements were accomplished by using Affymetrix 430 v0.2 arrays, and data are included only if the probeset produced a "present" response (i.e., hybridization above background) on all the arrays (23) used in this experiment. *P* values were calculated by contrasting group means within a one-way ANOVA and were adjusted for multiple testing within gender by using a FDR.

Gene Symbol	Affymetrix Probe ID	Female Mice						Male Mice					
		Adcy7(+/-)			Adcy7 ^{huTG}			Adcy7(+/-)			Adcy7 ^{huTG}		
		Percentage Change	Unadjusted <i>P</i> Value	FDR	Percentage Change	Unadjusted <i>P</i> Value	FDR	Percentage Change	Unadjusted <i>P</i> Value	FDR	Percentage Change	Unadjusted <i>P</i> Value	FDR
		%			%			%			%		
Adcy2	1455462_at	-5	0.451	0.650	-5	0.520	0.817	7	0.271	0.541	-1	0.828	0.876
Adcy3	1421960_at	14	0.027	0.293	10	0.135	0.744	-19	0.001	0.010	-23	<0.001	0.002
Adcy6	1418128_at	2	0.866	0.866	7	0.514	0.817	3	0.773	0.817	-2	0.810	0.876
Adcy7	1450065_at	-14	0.473	0.296	-17	0.438	0.916	-23	0.229	0.916	10	0.643	0.876
Adcy7	1456307_s_at	-35	0.054	0.650	-9	0.670	0.817	-20	0.290	0.817	3	0.876	0.876
Crhr1	1418810_at	2	0.788	0.866	-1	0.933	0.933	-16	0.060	0.933	5	0.555	0.876
Gnas	1450186_s_at	3	0.179	0.536	-3	0.337	0.780	0	0.921	0.921	-1	0.766	0.876
Gnas	1427789_s_at	-13	0.351	0.608	-30	0.042	0.374	-4	0.777	0.874	22	0.188	0.847
Pomc	1433800_a_at	3	0.411	0.650	1	0.775	0.916	-4	0.314	0.916	3	0.411	0.876

There were no significant differences in expression levels of *Adcy2* or *Adcy6* between *Adcy7^{huTG}* or *Adcy7(+/-)* and WT animals of either sex. *Adcy3* mRNA was significantly lower (by approximately 20%) in both male *Adcy7(+/-)* and *Adcy7^{huTG}* animals compared with WT littermates. As far as *Adcy7* mRNA was concerned, *Adcy7(+/-)* male and female animals displayed lower expression of the mouse *Adcy7* gene (mRNA) in the pituitary gland compared with their WT littermates. In male *Adcy7(+/-)* mice, *Adcy7* expression was approximately 20% lower than WT, whereas in female *Adcy7(+/-)* mice, *Adcy7* expression was 14 to 35% lower than WT, depending on the probe set. The results we obtained by measuring *Adcy7* gene expression by microarray technology were noted to demonstrate a large amount of biological variability, which precluded statistical significance, and thus we pursued further experiments to measure *Adcy7* mRNA by qRT-PCR.

Expression of the Human ADCY7 and Mouse Adcy7 mRNA in the Anterior Pituitary Gland Assessed by qRT-PCR. By qRT-PCR, mouse *Adcy7* mRNA levels in anterior pituitary gland of *Adcy7(+/-)* mice were approximately half of those in WT animals (male, relative quantity 0.44, $P < 0.0001$; female, relative quantity 0.48, $P < 0.0001$). Mouse *Adcy7* mRNA levels in male *Adcy7^{huTG}* mice were similar to those in WT mice (relative quantity 1.14, $P < 0.128$), whereas mouse *Adcy7* mRNA levels were lower in female *Adcy7^{huTG}* mice compared with WT (relative quantity 0.77, $P = 0.007$). However, as measured by qRT-PCR with

primers specific for human *ADCY7* mRNA, the human *ADCY7* mRNA was expressed only in *Adcy7^{huTG}* male and female mice and added measurably to the total *ADCY7* mRNA in anterior pituitary of the transgenic mice.

Expression of AC7 Protein in Whole Pituitary Gland. To analyze AC7 protein content in the mouse pituitary, we used an antibody designed to recognize both human (transgene) and mouse AC7 protein. There was a significant reduction (male, 24% lower, $P = 0.030$; female, 27% lower, $P < 0.0001$) in AC7 protein in the *Adcy7(+/-)* animals compared with WT (Fig. 2). The expression of the human transgene in the *Adcy7^{huTG}* mice resulted in a significant increase in the total AC7 protein content in anterior pituitary tissue. AC7 protein levels in *Adcy7^{huTG}* mice were 31% higher ($P = 0.013$) than WT levels in males and 45% higher ($P = 0.0007$) than WT levels in females (Fig. 1).

Blood Alcohol Levels. There was no difference in blood alcohol levels among WT, *Adcy7^{huTG}*, and *Adcy7(+/-)* groups of animals, of either sex, after the injection of 2.25 g/kg of ethanol at any time point. There was also no difference in blood alcohol levels between male and female animals at any of the time points measured [three-way ANOVA (genotype, sex, and time)] (Fig. 2).

Injection Effect and Baseline Adrenocorticotropin Levels. Table 2 displays the mean adrenocorticotropin levels at baseline (0 time after ethanol injection) and 15 min after saline injection. The 10-s exposure to CO₂ before decapitation has previously been shown to have no effect

Fig. 1. Protein levels of AC7 in pituitary gland. Levels of AC7 in whole pituitary glands of male (A) and female (B) *Adcy7^{huTG}*, *Adcy7(+/-)*, and WT mice were determined by immunoblotting as described under *Materials and Methods*. Each blot included tissue from male or female WT, *Adcy7^{huTG}*, and/or *Adcy7(+/-)* mice, and the level of AC7 protein, quantitated as described in the text, was normalized to β -tubulin and expressed as percentage of WT on each blot. Percentage values were averaged across blots. Data are expressed as the mean $\pm$ S.E.M. from three to nine mice per group. *, $P < 0.02$ versus WT (two-way ANOVA and post-hoc contrasts). Representative blots are shown.

Fig. 2. Time course of blood ethanol levels after ethanol injection. Male (A) and female (B) WT (○), Adcy7^{huTG} (▼), and Adcy7(+/-) (●) mice were injected intraperitoneally with 2.25 g/kg ethanol, and blood ethanol levels were measured by head-space gas chromatography at the indicated times after injection. Values represent mean $\pm$ S.E.M. from four to nine mice per group at each time point.

TABLE 2

Adrenocorticotropin levels at baseline and 15 min after saline injection

Units of adrenocorticotropin are pg/ml plasma. Means and S.E. were calculated by using the least-squares method in a three-way ANOVA model. There is a significant difference between males and females at baseline ($P = 0.012$) in all genotypes. There was also a significant effect of saline injection in males ($P < 0.001$) and females ($P = 0.001$) of all genotypes.

Genotype	Female Mice				Male Mice			
	Baseline		After Saline Injection		Baseline		After Saline Injection	
	<i>n</i>	Mean (S.E.)	<i>n</i>	Mean (S.E.)	<i>n</i>	Mean (S.E.)	<i>n</i>	Mean (S.E.)
Adcy7(+/-)	4	21.7 (4.2)	4	45.1 (4.2)	7	11.3 (3.1)	4	28.8 (4.2)
Wild type	6	27.0 (3.4)	8	34.5 (2.9)	9	11.5 (2.8)	6	33.0 (3.4)
Adcy7 ^{huTG}	6	23.9 (3.4)	5	36.0 (3.7)	6	16.8 (3.4)	3	37.4 (4.8)

on plasma adrenocorticotropin levels (Reed et al., 2009). At baseline, there were no significant differences among genotypes in either males or females. However, there was a significant difference between adrenocorticotropin levels in male and female mice, per se, at baseline ($F_{3,56} = 6.25$, $P = 0.012$), with adrenocorticotropin levels being higher in females. The biggest sex effect was in the wild-type mice (difference between females and males, 15.5 ± 4.4 pg/ml plasma), and the smallest sex effect was in the transgenic mice (7.1 ± 4.8 pg/ml plasma). When adrenocorticotropin levels were measured at 15 min after saline injection, there were no significant differences among genotypes in males or females, but the rank order of adrenocorticotropin levels in males was Adcy7^{huTG} > WT > Adcy7(+/-), whereas in females, the rank order was WT > Adcy7^{huTG} > Adcy7(+/-). There was no significant difference in adrenocorticotropin levels between males and females at this time.

There was, however, a significant injection effect (i.e., adrenocorticotropin levels 15 min after saline injection minus adrenocorticotropin levels at baseline) in both males ($F_{3,56} = 15.90$, $P < 0.001$) and females ($F_{3,56} = 8.14$, $P = 0.001$). The injection effect did not differ among genotypes or between males and females.

Baseline Corticosterone Levels. Baseline corticosterone levels did not differ significantly among genotypes. Also there were no significant differences between sexes in the Adcy7(+/-) or Adcy7^{huTG} mice. However, WT female mice

had significantly higher corticosterone levels than WT male mice ($t = 3.65$, $P = 0.0004$).

Adrenocorticotropin Levels after Ethanol Injection: Genotype Effects. Figure 3 shows adrenocorticotropin levels in the three genotypes at baseline (0 time) and four time points after ethanol injection for male and female mice. Ethanol treatment increased adrenocorticotropin levels in all groups of mice ($F_{24,153} = 15.50$, $P < 0.001$) at 15, 30, and 45 min after injection, with a peak at 15 min after injection. The ANOVA model indicated a significant three-way interaction between effects of genotype, sex, and time on adrenocorticotropin levels. The analyses within time points indicated significant effects of genotype and/or sex on adrenocorticotropin levels at 15 min ($F_{5,153} = 6.31$, $P < 0.0001$), 45 min ($F_{5,153} = 7.35$, $P < 0.0001$), and 75 min after ethanol injection ($F_{5,153} = 9.52$, $P < 0.0001$).

At 15 min after ethanol injection, there was a significant difference in adrenocorticotropin levels among genotypes in males ($F_{2,153} = 7.59$, $P = 0.001$). Male Adcy7^{huTG} mice had higher adrenocorticotropin levels than wild type ($t = 2.72$, $P = 0.007$) and Adcy7(+/-) male mice ($t = 3.89$, $P < 0.001$). There was also a significant difference among genotypes in female mice at 15 min after ethanol injection ($F_{2,153} = 3.66$, $P = 0.03$). Female Adcy7^{huTG} mice had higher adrenocorticotropin levels than Adcy7(+/-) mice ($t = 2.60$, $P = 0.01$).

At 45 min after ethanol injection, there was a significant difference in adrenocorticotropin levels among genotypes in females ($F_{2,153} = 6.22$, $P = 0.003$). The female Adcy7^{huTG}

Fig. 4. Area under the adrenocorticotropin level $\times$ time curve after ethanol injection. Data shown in Fig. 3 were used to calculate AUC for male and female Adcy7^{huTG}, WT, and Adcy7(+/-) mice. Data are expressed as mean $\pm$ S.E.M. *, $P < 0.01$ (males), $P < 0.003$ (females), Adcy7^{huTG} compared with Adcy7(+/-) mice. †, $P = 0.016$, male WT compared with male Adcy7(+/-) mice. The AUC for female Adcy7(+/-) mice is significantly higher than that for male Adcy7(+/-) mice (see text).

mice had higher adrenocorticotropin levels than Adcy7(+/-) mice ($t = 3.12$, $P = 0.002$). The genotype effect in males at this time only marginally missed the significance threshold ($F_{2,153} = 3.00$, $P = 0.053$).

To obtain a more integrated assessment of the effect of ethanol on adrenocorticotropin levels, we calculated AUC for adrenocorticotropin levels over time after injection (Fig. 4). Ethanol treatment significantly increased the AUC for all groups of mice ($p < 0.05$). The AUC was significantly higher in the Adcy7^{huTG} mice than in the Adcy7(+/-) mice of both sexes (males: $t = 4.56$, $df = 17.84$, $P < 0.001$; females: $t = 3.32$, $df = 24.05$, $P = 0.003$). In addition, the wild-type male mice had a significantly higher AUC than the Adcy7(+/-) male mice ($t = 2.61$, $df = 21.54$, $P = 0.016$).

Adrenocorticotropin Levels after Ethanol Injection: Effect of Sex. There were differences in adrenocorticotropin levels between males and females after ethanol injection at 15 min ($F_{3,153} = 4.90$, $P = 0.003$), 45 min ($F_{3,153} = 5.42$, $P = 0.001$), and 75 min ($F_{3,153} = 11.15$, $P < 0.001$). At 15 min after ethanol injection, male Adcy7(+/-) and WT mice had lower adrenocorticotropin levels than their female counterparts ($t = 3.06$, $P = 0.003$; $t = 2.30$, $P = 0.023$, respectively).

Fig. 3. Effect of ethanol on plasma adrenocorticotropin levels. Male (left) and female (right) WT (red ■), Adcy7^{huTG} (blue ♦), and Adcy7(+/-) (green ▲) mice were injected intraperitoneally with 2.25 g/kg ethanol and killed at the time points indicated. Adrenocorticotropin was measured by using an enzyme immunoassay kit as described in the text. Data are expressed as mean $\pm$ S.E.M. from four to eleven mice per group. *, $P = 0.007$, male Adcy7^{huTG} compared with WT mice, and $P < 0.001$ compared with Adcy7(+/-) mice; **, $P < 0.01$, female Adcy7^{huTG} compared with female Adcy7(+/-) mice; †, $P < 0.002$, female Adcy7^{huTG} compared with Adcy7(+/-) mice. At 15, 45, and 75 min after ethanol injection, some groups of females had significantly higher adrenocorticotropin levels than males (see text).

Likewise, at 45 min after injection, male Adcy7(+/-) mice had lower adrenocorticotropin levels than female Adcy7(+/-) mice ($t = 4.01$, $P < 0.001$). At 75 min after ethanol injection, male Adcy7^{huTG} and Adcy7(+/-) mice had lower adrenocorticotropin levels than females of their respective genotype ($t = 3.40$, $P < 0.001$; $t = 4.52$, $P < 0.001$, respectively).

In terms of AUC measures after ethanol injection, with regard to sex differences, male mice of all genotypes had a lower AUC for adrenocorticotropin than females of the same respective genotype, but this difference reached statistical significance only in the Adcy7(+/-) mice ($t = 5.89$, $df = 15.58$, $P < 0.001$).

Corticosterone Levels after Ethanol Injection: Genotype Effects. Figure 5 shows the corticosterone levels in the three genotypes at baseline (0 min) and the four time points after ethanol injection in male and female mice. Ethanol treatment resulted in increased corticosterone levels in all groups of mice ($F_{24,141} = 67.78$, $P < 0.001$) at all time points after injection, with peak levels seen at 30 to 45 min after injection. The ANOVA model indicated a significant three-way interaction between genotype, sex, and time on circulating corticosterone levels. A significant effect of genotype and/or sex was seen after ethanol injection at 15 min ($F_{5,141} = 138.81$, $P < 0.001$), 30 min ($F_{5,141} = 51.71$, $P < 0.001$), and 45 min ($F_{5,141} = 7.02$, $P < 0.001$).

At 30 min after ethanol injection, there was a significant genotype-dependent effect on corticosterone levels in females ($F_{2,141} = 31.86$, $P < 0.001$). The Adcy7^{huTG} female mice had higher corticosterone levels than the WT mice ($t = 3.72$, $P < 0.001$) and the Adcy7(+/-) mice ($t = 7.87$, $P < 0.001$). In addition, the female WT mice had significantly higher corticosterone levels than the Adcy7(+/-) mice ($t = 2.98$, $P = 0.003$). Although the genotype effect was not significant in the male mice at 30 min after injection, the rank order of corticosterone levels in male mice of the different genotypes was similar to that in females at this time. At 45 min after ethanol injection, there was a significant difference among genotypes in male mice ($F_{2,141} = 3.19$, $P = 0.003$). The Adcy7(+/-) male mice had lower corticosterone levels than the WT mice ($t = 2.28$, $P = 0.024$) and the Adcy7^{huTG} mice ($t = 3.51$, $P < 0.001$). The rank order of corticosterone levels across genotypes in females was similar to that in males at this time point.

We also calculated the AUC for corticosterone levels over time after ethanol injection (Fig. 6). Results similar to those

Fig. 6. Area under the corticosterone level $\times$ time curve after ethanol injection. Data shown in Fig. 5 were used to calculate AUC for male and female Adcy7^{huTG}, WT, and Adcy7(+/-) mice. Data are expressed as mean $\pm$ S.E.M. *, $P = 0.036$ (males) and 0.011 (females), compared with Adcy7(+/-) mice. Females of all genotypes had significantly higher AUCs than males (see text).

seen in the AUC analysis of adrenocorticotropin were evident, i.e., ethanol treatment significantly increased the AUC in all groups of mice ($p < 0.0002$), and the Adcy7^{huTG} mice had a significantly higher AUC than the Adcy7(+/-) mice in both males and females ($t = 2.46$, $df = 9.12$, $P = 0.036$ and $t = 2.93$, $df = 14.62$, $P = 0.011$, respectively).

Corticosterone Levels after Ethanol Injection: Effect of Sex. There was a significant difference in corticosterone levels between males and females after ethanol injection at 15 min ($F_{3,141} = 116.77$, $P < 0.001$), 30 min ($F_{3,141} = 56.74$, $P < 0.001$), and 45 min ($F_{3,141} = 7.98$, $P < 0.001$). At 15 min, levels in females were higher than levels in males in all three genotypes [Adcy7(+/-): $t = 10.18$, $P < 0.001$; Adcy7^{huTG}: $t = 7.57$, $P < 0.001$; WT: $t = 13.76$, $P < 0.001$]. Again at 30 min, in all three genotypes, females had higher corticosterone levels than males [Adcy7(+/-): $t = 4.03$, $P < 0.001$; Adcy7^{huTG}: $t = 10.59$, $P < 0.0001$; WT: $t = 6.47$, $P < 0.001$]. At this time, there was also a significant difference in sex effects among the genotypes ($F_{2,141} = 6.61$, $P = 0.002$), i.e., the greatest difference in corticosterone levels between males and females occurred in the Adcy7^{huTG} mice, and the smallest difference occurred in the Adcy7(+/-) mice. Finally, at 45 min after ethanol injection, corticosterone levels were again higher in females than males within all three genotypes [Adcy7(+/-): $t = 2.33$, $P = 0.022$; Adcy7^{huTG}: $t = 3.76$, $P < 0.001$; WT $t = 2.10$, $P = 0.038$].

Fig. 5. Effect of ethanol on plasma corticosterone levels. Male (left) and female (right) WT (red $\circ$), Adcy7^{huTG} (blue $\diamond$), and Adcy7(+/-) (green $\blacktriangle$) mice were injected intraperitoneally with 2.25 g/kg ethanol and killed at the indicated times after injection. Plasma corticosterone levels were measured by using an enzyme-linked immunosorbent assay kit, as described in the text. Data are presented as mean $\pm$ S.E.M. from four to nine mice per group. *, $P = 0.001$ compared with female WT and Adcy7(+/-) mice; **, $P < 0.003$ compared with female Adcy7(+/-) mice. †, $P = 0.024$ compared with male WT mice, $P < 0.001$ compared with male Adcy7^{huTG} mice. For all three genotypes, females had significantly higher corticosterone levels than males at 15, 30, and 45 min after ethanol injection (see text).

Within all three genotypes, females had a significantly higher AUC for corticosterone than males [Adcy7^{huTG}: $t = 6.31$, $df = 9.92$, $P < 0.001$; wild type: $t = 4.51$, $df = 12.76$, $P < 0.001$; Adcy7(+/-): $t = 3.58$, $df = 10.34$, $P = 0.005$] after injection of ethanol.

Discussion

The critical tools in our demonstration of the involvement of AC7 in pituitary adrenocorticotropin release were the Adcy7(+/-) and Adcy7^{huTG} mice created to underexpress and overexpress AC7 in brain. Although our previous work generated information on brain gene expression in these mice (Hines et al., 2006), we felt it necessary to examine pituitary gene expression in the animals being used in the current studies. The microarray data that we obtained can be accessed on <http://phenogen.ucdenver.edu>. Using the criteria described in *Results*, we found that mRNA for *Adcy2*, *Adcy3*, *Adcy6*, and *Adcy7* is expressed in pituitary. One would assume that *Adcy9* mRNA is expressed in rodent corticotrophs, from the work of Antoni et al. (2003), although, based on our microarray data, the level of expression (mRNA) may be low in mouse. In the microarray experiment, only *Adcy3* mRNA was significantly different in the genetically modified mice, i.e., this mRNA was lower in both the Adcy7(+/-) and the Adcy7^{huTG} male mice compared with their WT littermates. With regard to our work, however, the differences in *Adcy3* mRNA, even if they are reflected by differences in AC3 protein localized in corticotrophs, would not translate into ethanol-induced changes in cAMP production. We have shown that under several conditions AC3 is unresponsive to ethanol, even at concentrations as high as 200 mM (Yoshimura and Tabakoff, 1995).

The results of the qRT-PCR analysis in the Adcy7(+/-) mice mirrored qualitatively the results of the microarray analysis, but were more consistent and demonstrated a statistically significant, approximately 50% decrease in the mouse *Adcy7* mRNA in both male and female Adcy7(+/-) mice compared with WT. The qRT-PCR for mouse *Adcy7* mRNA, and the gene array analyses, did not quantitate the product of the human AC7 transgene that was introduced into the oocyte during the creation of the Adcy7^{huTG} strains. The additional expression of the human AC7 in the pituitary of the Adcy7^{huTG} mice was, however, demonstrated by using primers specific for the human *ADCY7* mRNA. The results obtained with an antibody that did not distinguish between

the mouse and human AC7 substantiated lower AC7 protein levels in anterior pituitary of the *Adcy7*(+/-) male and female mice and increased levels in *Adcy7*^{huTG} male and female mice compared with the WT littermates.

Evidence has been presented that in G_s -coupled systems the AC component may be rate-limiting in the receptor- G_s -AC signal transduction event (i.e., generation of cAMP) (MacEwan et al., 1996). Thus, if signaling through AC7 is important in a physiologic event, one would expect that increasing or decreasing the levels of AC7 may produce related changes in the measured physiologic event. The baseline levels of adrenocorticotropin in the circulation of our male and female mice are comparable with levels reported by others (Lolait et al., 2007; Carter et al., 2009). Fifteen minutes after a "mild" stress of saline injection, adrenocorticotropin levels in plasma increased in both male and female mice. The rank order of increase in male mice [*Adcy7*(+/-) < WT < *Adcy7*^{huTG}] followed the pattern expected from the measured levels of AC7 in male pituitaries. The female mice, however, did not illustrate a rank order expected from the genetic modification of their pituitary AC7 levels. One explanation of this observation is that the saline injection increased the adrenocorticotropin levels 2-fold or less in all groups of females, whereas in males the increase was more than 2-fold in all groups. The saline injection stimulus may not have been sufficient to fully engage the AC7 signaling in females.

As already noted, ethanol can potentiate the activation of AC7 by G_s -coupled receptors (e.g., CRF-R1), without the intervention of other stress-related receptor systems (e.g., AVP-R1b) (Tabakoff et al., 2001; Nelson et al., 2003). The *Adcy7*^{huTG}, *Adcy7*(+/-), and WT animals (male or female) in our studies did not differ significantly either in CRF-R1 mRNA or $G_s\alpha$ mRNA in pituitary. As noted in the Introduction, although ethanol can increase CRF release from the paraventricular nucleus of the hypothalamus both in vivo (Lee et al., 2004) and in vitro (Li et al., 2005), the increases do not correspond, in time, with the increase in adrenocorticotropin after ethanol treatment witnessed in our studies. A parsimonious explanation of our results would be that ethanol rapidly increases adrenocorticotropin levels in the circulation by promoting the response of AC7 to CRF receptor activation, even at constant levels of CRF. The increased cAMP then activates the mechanisms for adrenocorticotropin release (Aguilera et al., 1983; Sobel, 1985; Wynn et al., 1985). Our previous work using cultured cell systems indicates that significant increases in AC7 activity in response to $G_s\alpha$ -coupled receptor stimulation occur at concentrations of 20 mM ethanol, and the 2.25 g/kg dose we administered produced blood ethanol concentrations of above 40 mM.

In both males and females, the increase in adrenocorticotropin after ethanol injection was proportional to the pituitary levels of AC7, which was particularly notable when the AUC of the time/response curves for adrenocorticotropin were compared among the *Adcy7*(+/-), WT, and *Adcy7*^{huTG} mice. Examination of the blood ethanol concentrations over the time of measurement of adrenocorticotropin indicated that whereas adrenocorticotropin dropped to basal levels within 60 to 80 min after ethanol injection, the ethanol levels in plasma diminished little over this time period. Our previous studies with AC7 in human erythroleukemia cells demonstrated that the ethanol stimulation of cAMP production

"desensitized" significantly after a 30-min preincubation with ethanol (Rabbani et al., 1999), and this phenomenon may well be operational in the mouse pituitary corticotrophs.

There were significant quantitative differences in both peak levels and the AUC of the corticosterone concentration/time curves, which correlated in magnitude with the plasma levels of adrenocorticotropin in the *Adcy7*(+/-), WT, and *Adcy7*^{huTG} mice given ethanol. The rank order of the corticosterone responses to ethanol was such that the *Adcy7*^{huTG} mice (male and female) demonstrated the highest peak and, particularly, greater AUC values, WT mice were intermediate, and the *Adcy7*(+/-) mice had the lowest peak and AUC values.

What is also noticeable in our results (see AUCs) are the significantly higher levels of corticosterone in the circulation of the female mice in comparison with the male mice, especially in the *Adcy7*^{huTG} mice, in whom levels of adrenocorticotropin after ethanol administration were not statistically different in males and females. These observations suggest that the adrenal tissue of the females may be more responsive to high levels of adrenocorticotropin than that of males. Nevertheless, our work indicates that by manipulating AC7 levels in the pituitary one can alter the magnitude of an animal's adrenocorticotropin response to ethanol and possibly to stress (e.g., intraperitoneal injection of saline in male mice). The adrenocorticotropin in turn produces a graded response in circulating corticosterone levels in mice that is quantitatively related to the levels of AC7 in the pituitary.

The current work not only helps to establish AC7 as an important part of the signaling cascade between the CRF-R1 and adrenocorticotropin release under conditions of ethanol intoxication and/or stress, but also allows some extrapolation to situations witnessed with humans. Our previous work with AC7 polymorphisms in humans demonstrated the association of a haplotype that includes the 3'-untranslated region of human *ADCY7* with familial major depressive disorder. This association was primarily evident in females (odds ratio = 2.0). If the same population was divided into women who drink alcohol at harmful levels (>40 g/day) and those who drink <40 g/day the odds ratio for the association of the relevant haplotype in *ADCY7* with "harmful" alcohol consumption, in women who also demonstrate familial depression, increased to 3.0. The polymorphic 3'-untranslated region in the *ADCY7* gene in humans contains elements that can alter mRNA transcription/stability/translation, including a tetranucleotide repeat region (Helleuo et al., 1997) and a nearby polymorphic miRNA binding site. These sequence differences may promote different levels of expression of *ADCY7* among individuals, making certain individuals more susceptible to increase their circulating levels of adrenocorticotropin and cortisol after alcohol consumption or even emotional stress. There is certainly a plethora of evidence linking high levels of circulating glucocorticoids to major depressive disorder (Pfohl et al., 1985; Mitchell and O'Keane, 1998; Vreeburg et al., 2009). Thus the demonstration in this article that AC7 levels in the pituitary influence adrenocorticotropin and glucocorticoid levels in the circulation generates a testable mechanistic hypothesis regarding *ADCY7* polymorphisms, environmental events (including ethanol consumption), and the etiology of major depressive disorder in humans (particularly in women).

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